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Genetic Control of Signal Transduction in Mouse Melanocytes

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At the final step of melanocyte differentiation in mouse hair follicles, the cells produce melanin. The type of melanin they produce is, however, determined by the tissue environment of hair follicles. In wild-type mice, melanocytes located in hair bulbs synthesize eumelanin at the beginning of hair growth. They subsequently produce pheomelanin and finally produce eumelanin again. Therefore, the hair is characterized by a subterminal band of yellow, with the rest of it displaying black. This characteristic is called the agouti pattern and is known to be determined by the wild-type allele, *A* at the *a* (agouti) locus, which is considered to function in the follicular cells. Expression of the agouti pattern is altered by genetic substitutions at the *a* locus and the *e* (extension) locus. Animals heterozygous for the A^y (lethal yellow) allele exhibit yellow coat color; those homozygous for the *e* (recessive yellow) also produce yellow hair exclusively.

By using an organ culture method, we demonstrated that α-MSH and cholera toxin, as well as forskolin, induced eumelanin synthesis in explants from lethal yellow mice (A^y/a). On the other hand, these reagents did not induce eumelanogenesis in the hair follicles of recessive yellow (e/e) mice. Therefore, we assume that the product of the *a* locus, which probably functions in follicle cells, interacts with α-MSH at the α-MSH receptor and that the *e* locus controls the functionality of adenylate cyclase in the membrane of mouse melanocytes. *J Invest Dermatol 92:239S–242S, 1989*

A signal transduction mechanism in the cell membrane, which receives information from the cellular environment and transfers it to the site of cellular function, has been demonstrated in various cell types [1,2]. Tamate and Takeuchi [3,4] have shown that the switch between eumelanin and pheomelanin synthesis, which results in the pattern formation of mouse hair, involves a signal transduction mechanism.

In wild-type mice, melanocytes located in hair follicles produce eumelanin at the beginning of hair growth. They subsequently produce pheomelanin, and finally produce eumelanin again. Therefore, their hair is characterized by a subterminal band of yellow, with the rest of it showing black because melanosomes formed in melanocytes are constantly transferred to keratinocytes. This characteristic is called the agouti pattern and is known to be determined by the *A* allele, or wild-type allele at the *a* (agouti) locus.

Previous studies by Silvers and Russell [5] have shown that the genes at the *a* locus act via the follicular environment (Silvers and Russell, 1955). They grafted intensely pigmented black mice of the genotype *a/a;C/c* shortly after birth with the skin of albino mice of the genotype $A^y/a;c/c$ and found some intensely colored yellow hair among the nonpigmented hairs within the border of the graft. Their result indicated that the kind of melanin synthesized by the invading cell was not determined by the genotypes of the cells but the genotypes of the receiving hair follicles. In other words, the genes at the agouti locus produce their effect by determining follicular environment.

On the other hand, Geschwind and his colleagues [6,7] demonstrated that MSH (melanocyte stimulating hormone) exhibited an inhibitory action on pheomelanogenesis. They induced eumelanin synthesis in pheomelanin producing hair-follicle melanocytes by injecting the lethal yellow mouse with α-MSH. Subsequently, Tamate and Takeuchi [3,4] were able to examine the effect of α-MSH on the hair-follicle melanocytes of pheomelanic genotypes in vitro and reported the induction of eumelanogenesis by α-MSH in the skin explants from the lethal yellow mice (A^y/a). They found numerous eumelanosomes beside intrinsic mature pheomelanosomes in the hair-follicle melanocytes which darkened after α-MSH treatment. They also induced eumelanin formation in the hair follicle melanocytes of the lethal yellow with dibutyryl cyclic adenosine monophosphate (dbcAMP) and assumed that the eumelanin induction by α-MSH in the lethal yellow follicles was mediated by cAMP and that the product of the agouti locus primarily interacted and probably competed with the α-MSH-cAMP signal tranduction system in the membrane.

Another mutation, *e* (recessive yellow), at the *e* locus, which gives rise to pheomelanin formation, was also shown to be involved in the signal transduction mechanism. Tamate and Takeuchi [4] reported that in their in vitro study eumelanogenesis was not induced by α-MSH in the recessive yellow melanocytes, whereas the skin explants exposed to dbcAMP were found to contain eumelanin. These results indicate that the action of α-MSH on the lethal yellow melanocytes is mediated by cAMP as the second messenger, and that the melanocyte of recessive yellow mouse has a deficiency in responding to α-MSH. It seems probable that the *e* locus controls a step in the signal transduction mechanism in the membrane of melanocytes. An attempt was made in the present study to pinpoint the site of action of the *e* locus in the signal-transducing process in the membrane by using cholera toxin that stimulates GTP-binding protein and forskolin, which is considered to be a potent activator of membrane-bound adenylate cyclase.

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Abbreviations:
dbcAMP: dibutyryl cyclic adenosine monophosphate
MSH: melanocyte stimulating hormone

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MATERIALS AND METHODS

Mice used in the present study were C57BL/6J-A^y (genotype A^y/a) and C57BL/6J-*e* (genotype *e/e*) provided by the Jackson Laboratory, Bar Harbor, Maine. The A^y (lethal yellow) is a dominant allele at the *a* locus and exhibits yellow coat color. The *e* (recessive yellow) is a recessive allele at the *e* (extension) locus and also exhibits yellow coat color. In the both genotypes, melanocytes located in hair bulbs solely produce pheomelanin.

Skin explants (approximately 1 × 1 mm) were excised from the dorsum of 7.5-day-old mice, rinsed three times with Hanks' balanced salt solution (BSS), and cultured according to the method described previously by Takeuchi et al [8]. Cholera toxin (Sigma) or forskolin (Hoechst) at various concentrations was added to the culture medium, Ham 12, containing 2% fetal bovine serum.

For electron microscopy, the explants were doubly fixed with 3.5% glutaraldebyde in 0.1 M phosphate buffer, pH 7.4, and 1% osmium tetroxide in the phosphate buffer, dehydrated, and embedded in Epon 812 (Shell Chemical Co., Atlanta, Georgia). After sectioning, samples were stained with uranyl acetate and lead citrate, then examined in a Hitachi H-500 electron microscope.

RESULTS

Effect of Cholera Toxin In the hair-follicle melanocytes of the skin explants from the lethal yellow mice (A^y/a), which were cultured for 2 d in the medium containing 10^{-8}–10^{-10} M cholera toxin, very dark pigments (presumably eumelanin) were observed. The mean frequency of eumelanin-containing hair follicles was 28.3% at a cholera toxin concentration of 10^{-9} M, significantly greater than that of the control culture ($P < 0.01$). The frequency of the eumelanin-producing hair follicles increased in cultures treated with 10^{-8} M cholera toxin to 62.9% (Fig 1). Although the 7.5-d-old skin contained some eumelanin containing hair follicles (8.0%), there was only a slight increase in 2-d cultures. On the other hand, in the recessive yellow mice (*e/e*) no increase in the frequency of eumelanin-containing melanocytes was observed in hair follicles cultured in medium containing cholera toxin at the concentrations tested.

Effect of Forskolin In both genotypes (A^y/a and *e/e*), hair-follicle melanocytes from 7.5-d-old mice exclusively contained pheomelanin. In 2-d cultures, a few hair bulbs in the control explants were darkened. The frequency was 2.84% for A^y/a, while it was 0.36% for *e/e*. However, in hair follicles cultured in the forskolin containing medium significant darkening was observed; the frequency of darkened hair follicles was 27.0% with 1.0×10^{-6} M, 57.72% with 1.0×10^{-5} M, and 44.0% with 1.0×10^{-4} M forskolin. These frequencies were significantly higher than that of the control ($P < 0.05$–$P < 0.01$) (Fig 2).

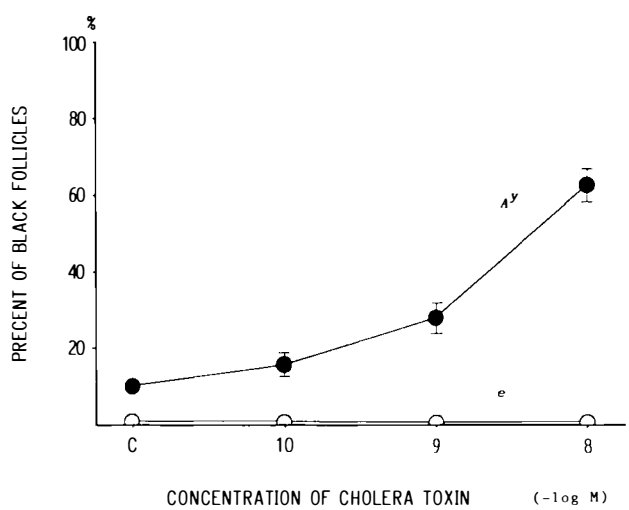

Figure 1. Effect of cholera toxin on the induction of eumelanogenesis in pheomelanic hair follicles with the genotypes A^y/a (A^y) and *e/e* (*e*). Each value is the mean ± standard error from 12 explants in A^y/a and eight explants in *e/e*. C: control.

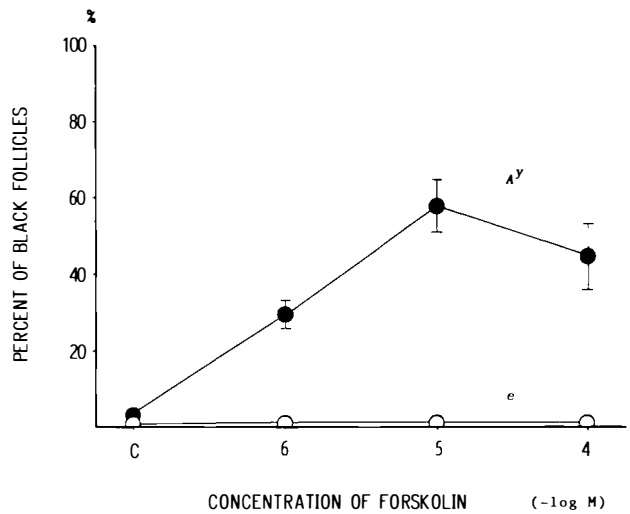

Figure 2. Effect of forskolin on the induction of eumelanogenesis in pheomelanic hair follicles with the genotypes A^y/a (A^y) and *e/e* (*e*). Each value is the mean ± standard error from eight explants in A^y/a and 11 explants in *e/e*. C: control.

On the other hand, when skin explants with *e/e* genotypes were cultured in the forskolin-containing medium (1.0×10^{-4} M, 1.0×10^{-5} M, 1.0×10^{-6} M), the frequencies of darkening of hair follicles were low (0.72–1.09%) and were not significantly different from that of the control culture (Fig 2).

In order to identify the type of melanosome in the darkened hair follicles, the ultrastructure of the hair-follicle melanocyte in the explants was observed with an electron microscopy. In the hair-follicle melanocytes with the genotype A^y/a cultured in control medium, spherical melanosomes, characteristic of pheomelanosomes, were observed (Fig 3*a*). In contrast, premelanosomes of eumelanosomal type were observed in addition to mature pheomelanosomes in the hair-follicle melanocytes with A^y/a genotype cultured for 2 d in forskolin-containing medium (Fig 3*b*). These premelanosomes largely contained striated longitudinal matrix, characteristic of eumelanosomes. Therefore, the type of melanin synthesized upon the treatment with forskolin appears to be eumelanin.

DISCUSSION

The agouti pattern formation in mice has evoked interests among developmental biologists and geneticists since the discovery by Silvers and Russell [5] that the gene responsible for the pattern formation does not act autonomously, but acts through the tissue environment. Thus, such genes (the wild-type allele, *A*, or the dominant allele, A^y, at the agouti locus) are expressed in cells (probably in hair follicles) which surround melanocytes and transfer their products to melanocytes. The mode of action of genes at the *a* (agouti) locus was unclear until Geschwind and co-workers [6,7] found that eumelanin formation could be induced in the agouti (A/A) or yellow (A^y/a) mice by injecting them with α-MSH, suggesting that α-MSH could be involved in the agouti pattern formation. Subsequently, Tamate and Takeuchi [3] demonstrated that cultured hair follicles with the genotype A^y/a respond to either α-MSH or DbcAMP to produce eumelanin and suggested that the product of the A^y gene was repressed by α-MSH, probably through competition for the α-MSH receptor. On the other hand, pheomelanic hair follicles with the genotype *e/e* did not respond to α-MSH but responded to DbcAMP [4]. These findings led them to assume that the *e* locus, where recessive allele produces yellow coat color when homozygous, regulates events distal to the α-MSH receptor.

An attempt was made in the present study to clarify the site of the gene action of the *e* locus in the signal transduction from α-MSH receptor to adenylate cyclase in mouse melanocytes. For this pur-

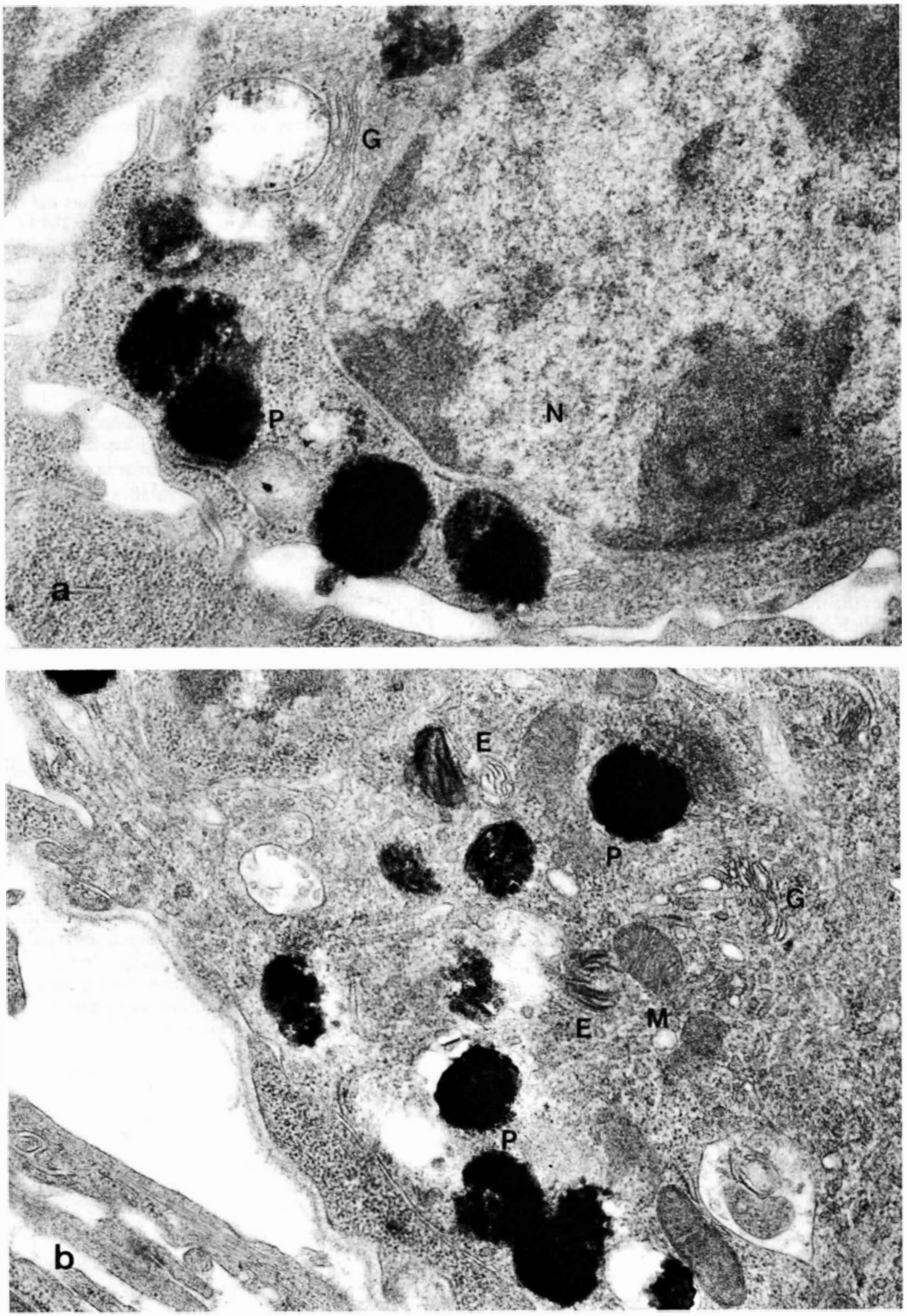

Figure 3. Electron micrographs of hair-follicle melanocytes from the explant cultured in the control medium *(a)* and in the explant cultured in the medium containing 10^{-5} M cholera toxin *(b)*. *E*: eumelanosome; *G*: Golgi apparatus; *M*: mitochondrion; N: nucleus; P: pheomelanosome (×30,000).

pose, cholera toxin [9] or forskolin [10] was added to culture medium.

In the lethal yellow (A^y/a) hair follicles, both cholera toxin and forskolin were shown to induce eumelanogenesis. Therefore, it is likely that the signal received by the α-MSH receptor is transduced to adenylate cyclase via a GTP protein in the membrane of mouse melanocytes.

In the recessive yellow (e/e) hair follicles, melanocytes did not respond to cholera toxin or forskolin, indicating that the site of control by the *e* locus was adenylate cyclase activity. Tamate and Takeuchi [4] suggested previously that the *e* locus controls a mechanism involved in the function of the α-MSH receptor. Here we suggest that the control point is the catalytic site, adenylate cyclase. This raises the question of whether or not the e/e mice express a deficiency in adenylate cyclase activity in cell types other than melanocytes. In the recessive yellow (e/e) mice, however, no pleio-

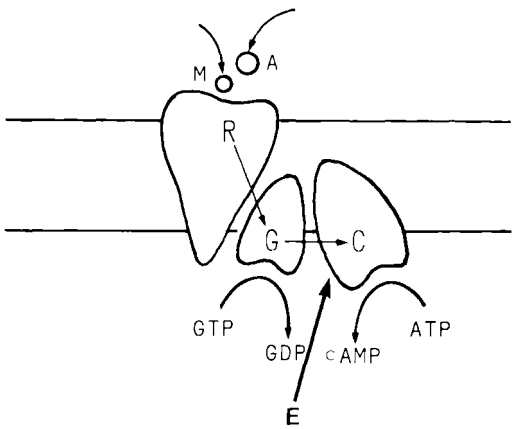

Figure 4. A model for the mechanism of signal transduction in the membrane of mouse melanocyte. *A*: the A factor, the product of the A^y, or *A* alleles at the *a* locus; *C*: adenylate cyclase; *E*: the product of the wild-type allele at the *e* locus; *G*: GTP-binding protein; *R*: α-MSH receptor.

tropic effect of the *e* gene has been reported. One possibility is that adenylate cyclase(s) has isozymes and differentiates depending on cell types. An alternative possibility is that *e* locus controls a melanocyte-specific regulating protein for adenylate cyclase(s).

Figure 4 illustrates the mechanism involved in the shift between eumelanogenesis and pheomelanogenesis in the mouse melanocyte and its genetic control. The signal provided by α-MSH is received by the α-MSH receptor on the membrane and is transduced to adenylate cyclase via a GTP-binding protein, which results in an increase in cAMP level in the cytoplasm. Eumelanin is synthesized under this condition. However, when the genotypes of follicular cells are A^y/a or A/A, the cells secrete the product of the *a* locus (the A factor), which competitively interacts with α-MSH, blocking the α-MSH receptor. Therefore, the level of cAMP in the melanocyte is lowered, giving rise to pheomelanogenesis. If the production of the A factor occurs periodically, melanocytes oscillate between eumelanogenesis and pheomelanogenesis, producing the agouti pattern. On the other hand, a genetic deficiency in adenylate cyclase activity leads to a lower cAMP level in the cytoplasm and results in pheomelanic melanocytes, as seen in the recessive yellow phenotype.

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